Genomic, Phenotypic, and Functional Analyses of T Cells in Patients with Psoriasis Undergoing Systemic Cyclosporin A Treatment

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Recent studies have demonstrated that cyclosporin A (CyA) exerts a beneficial effect on psoriasis. It remains unclear, however, whether T-cell immune responses are definitely impaired in psoriasis and whether the anti-psoriatic effect of CyA is mediated by interference with T-cell activation. To study these questions, 20 patients with severe psoriasis were treated with oral CyA (5 mg/kg/d) for 12 weeks and examined for several phenotypic and functional properties of peripheral blood T cells before and after therapy. The analyses included CD3, CD4, and CD8 phenotypes, IL-2 production and IL-2 receptor expression following Con A stimulation, proliferative responses to PHA, and in vivo responsiveness to a foreign antigen, PPD. When the values of patients before therapy and healthy individuals were compared, no statistically significant differences were detected in any of these analyses. Furthermore, none of these T-cell properties were changed after 12 weeks of treatment. To assess possible minor mutations in T-cell–related genes in psoriasis, the T-cell receptor β-chain locus was analyzed by Southern hybridization. With a cDNA probe for Cβ1, a polymorphic fragment of $\simeq$ 9 kb was detected in Eco RI digests in one of 20 patients and in four of 10 healthy individuals examined. No polymorphism was detected in Bam HI digests in any individual. These results fail to support the hypotheses that a general or "systemic" alteration in T-cell immunity plays a central role in the pathogenesis of psoriasis and in the action of CyA against this skin disorder. *J Invest Dermatol 96:376–382, 1991*

Psoriasis is a chronic skin disorder characterized by inflammation and abnormal epidermal proliferation as well as by a genetic predisposition. Many hypotheses have been postulated concerning its pathogenesis. These include impaired T-cell function [1–4]; imbalanced T-cell subsets ([5,6]; reviewed in [7]); HLA-DR and intercellular adhesion molecule-1 expressions on keratinocytes [8]; involvement of γ interferon/IP-10 [9]; alterations in protooncogene expression [10]; roles of growth regulatory factors such as epidermal growth factor [11], transforming growth factor-α [10,12], and IL-6 [13]; decreased β-adrenergic adenylate cyclase response [14]; elevated calmodulin levels [15]; abnormalities in arachidonic acid metabolism [16]; increased protease activity [17]; abnormalities in other cell types, such as antigen-presenting cells [18], fibroblasts [19], and endothelial cells [20]; chemotactic activities in the stratum corneum affecting neutrophil migration [21]; and neuropeptide action [22].

Since the beneficial effects of CyA on psoriasis have been demonstrated [23–28], many investigators have focused on a defect in the T cell as being the primary cause of the disease and as the primary target of CyA treatment, based chiefly on phenotypic analyses [27–30] and supported by the fact that CyA is a potent immunosuppressive agent, acting predominantly on T cells (reviewed in [31,32]). In addition, recent studies have revealed that CyA is effective in treating other abnormalities seen with psoriasis. CyA has been shown to possess direct antiproliferative effects on keratinocytes [33–36] as well as on fibroblasts [36] and endothelial cells [37], to inhibit EGF binding to keratinocytes [38], to augment the β-adrenergic response [39], to bind to calmodulin [40], to suppress phospholipase A2 activity [41], to inhibit Langerhans cell functions [42], and to suppress neutrophil chemotaxis [27,43]. This diversity of findings has made understanding the mechanism of action of CyA in psoriasis and the ultimate pathogenesis of this disease quite difficult.

Studies were conducted, therefore, to determine whether T-cell phenotypes and functions are indeed impaired in psoriasis and whether these activities are changed with CyA treatment by means of in vitro and in vivo analyses of each step in T-cell activation. The second purpose of this work was to analyze the possible existence of restriction fragment length polymorphism (RFLP) in the T-cell receptor (TcR) genes in psoriatic patients and to assess the frequency of RFLP in these patients as compared with healthy individuals.

MATERIALS AND METHODS

Patients Twenty patients (19 male and one female, 26 to 81 years old) with severe, active psoriasis were enrolled in this study, and the clinical characteristics of each patient are shown elsewhere [44]. Eighteen patients had only plaques, one had pustular psoriasis, and one had arthropathic psoriasis. The duration of disease ranged from 5 to 25 years, with a mean of 13.9 years. Severity of psoriasis, as expressed by the Psoriasis Area and Severity Index (PASI), ranged from 20.0 to 39.1, with a mean of 26.2 $\pm$ 5.9 before CyA treatment. Each patient received oral CyA in a dosage of 5 mg/kg/d for 12 weeks. Therapeutic efficacy was confirmed by the fact that the PASI

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Abbreviations:
CyA: cyclosporin A
FACS: fluorescent-activated cell sorter
IL-2R: interleukin-2 receptor
RFLP: restriction fragment length polymorphism
PASI: Psoriasis Area and Severity Index
PBL: peripheral blood lymphocytes
TcR: T-cell receptor

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decreased from 26.2 ± 5.9 before therapy to 5.1 ± 4.4 at 12 weeks. Blood levels of CyA were meausred by radioimmunoassay and were in the range of 40 to 300 ng/ml [44]. None of the patients received any other treatments except for one patient who received antihistamines throughout the time of this study. A heparinized venous blood sample was taken from, and intracutaneous PPD injection given to, each patient just before and at 12 weeks of treatment. Blood samples were analyzed within twelve hours.

Analysis of Lymphocyte Subsets Peripheral blood lymphocytes (PBL) prepared by Conray-Ficoll gradient (d = 1.077) were stained with a panel of FITC-labeled monoclonal antibodies against CD3, CD4, and CD8 (Coulter, Hialeah, FL). Cells were analyzed by a fluorescent-activated cell sorter (FACS) (EPICS-C, Coulter) to obtain the percentage of each phenotype. Total lymphocyte counts were determined by complete white blood cell count with differential.

Con A–Induced IL-2 Production PBL (2×10^5 cells/well) were cultured in triplicate in 96-well plates (Nunc, Denmark) for 24 h at 37°C in the presence of 10 μg/ml of Con A (E.Y. laboratory, San Mateo, CA) in an atmosphere of 100% humidity and 5% CO_2 in air. Culture medium was RPMI 1640 supplemented with 10% fetal calf serum, penicillin, streptomycin, and amikacin. Culture supernatants were assayed for IL-2 levels with a radioimmunoassay using ^{125}I-recombinant human IL-2 (Amersham, Buckinghamshire, UK), rabbit anti-human IL-2 (Eiken, Tokyo), and goat anti-rabbit IgG (Eiken). IL-2 level was expressed in U/ml using recombinant human IL-2 (Amersham) as a standard.

Con A–Induced IL-2 Receptor Expression PBL (1×10^6 cells/well) were cultured in 24-well plates for 48 h at 37°C in the presence of 10 μg/ml Con A. Cells were harvested by pipetting and stained with FITC-labeled anti-human IL-2 receptor (Coulter) followed by FACS analysis.

PHA Stimulation Assay PBL (1×10^5 cells/well) were cultured in triplicate in 96-well plates for 72 h at 37°C in the presence of 20 μg/ml PHA (Difco, Detroit, MI), which were added at the initiation of the culture period. Control cultures of unstimulated cells were maintained in parallel. Eight hours before harvesting, 0.25 μCi of ^{3}H-thymidine (Amersham) were added to each well. The cells were harvested with an automatic harvester and the radioactivity was determined with a liquid scintillation counter.

PPD Reaction The PPD reaction is commonly used to assess general cell-mediated immunity in Japan. Each patient received intracutaneous injection of 0.1 ml of PPD preparation (Japan BCG, Tokyo) on the flexure aspect of the forearm. The cutaneous reaction was evaluated after 48 h and expressed as the mean diameter of the erythematous area with infiltration.

Southern Hybridizations High-molecular-weight DNA was extracted from PBL by the proteinase K/phenol/chloroform/isoamyl alcohol method [45], and digested with 5 U/1 μg DNA of Eco RI or Bam HI (Sigma, St. Louis, Missouri). Digested DNA samples were electrophoresed in 1% agarose horizontal gels and transferred to a nylon membrane (Amersham) by the method of Southern [46]. Membranes were prehybridized for 5 h at 42°C and hybridized overnight at 42°C with a ^{32}P-labeled cDNA of the C1 region of the T-cell receptor β-chain (Cβ1) (P1020-^{32}P, Oncor, Gaithersburg, MD). Membranes were washed extensively and exposed to Kodak XR-5 film with intensifying screens at −70°C for 6 d.

Statistical Analysis All the data were expressed as the mean ± SD, and statistical differences were analyzed according to a paired t test or Student t test.

RESULTS

Each of the 20 patients showed significant improvement or complete clearance of psoriatic eruptions in response to CyA administration. There were, however, no significant changes in the total number of T cells or in T-cell subsets in peripheral blood taken before and after treatment. Total lymphocyte counts were 2.43 ± 0.86 (10^3 cells/mm^3) before therapy and 2.74 ± 0.70 after 12 weeks of CyA treatment. The percentage of CD3+ cells remained unchanged during the treatment: 71.1 ± 10.0% before therapy and 74.9 ± 7.9% after therapy (Fig 1). Although patient-to-patient variations were observed, the average percentage of CD4+ cells, which are considered the major target of CyA, was not reduced by CyA therapy: 41.0 ± 9.7% before therapy and 44.9 ± 6.7% after therapy (Fig 2). Nor was there a reduction in the percentage of CD8+ cells: 28.1 ± 8.8% before therapy and 30.5 ± 9.4% after therapy (Fig 3). In addition, the total number of lymphocytes and the phenotypic subsets of T cells in psoriasis patients displayed the same degree of variation as in the healthy volunteers. These results strongly suggest that CyA improved psoriasis without affecting the number or phenotype of peripheral blood T cells under the conditions used in this study.

To determine whether T-cell function(s) might be impaired in

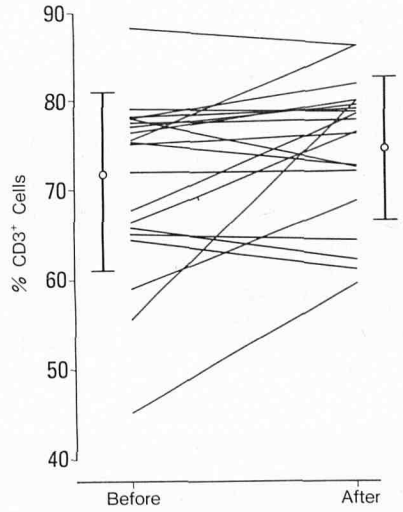

Figure 1. Percentages of CD3+ cells in PBL before and after CyA therapy. The percent CD3+ cells in PBL are plotted individually or as the mean ± SD (○) before and after 12 weeks of CyA therapy. The normal value is 65.9 ± 9.6 based on analyses of 21 healthy individuals.

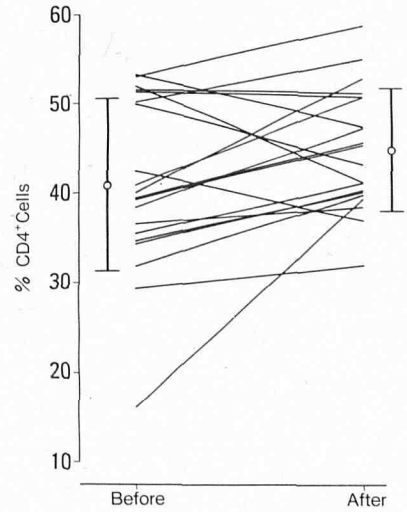

Figure 2. Percentages of CD4+ cells in PBL before and after CyA therapy. The percent CD4+ cells in PBL are plotted individually or as the mean ± SD (○) before and after 12 weeks of CyA therapy. The normal value is 42.6 ± 11.9 based on analyses of 20 healthy individuals.

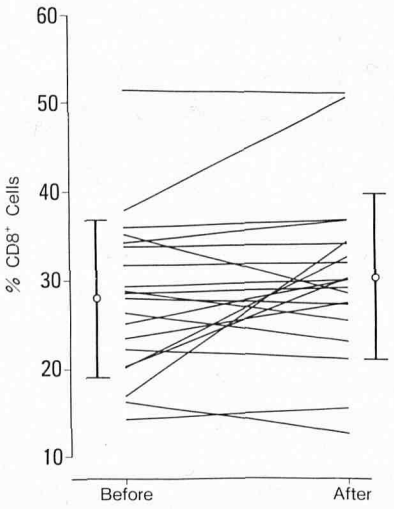

Figure 3. Percentages of CD8+ cells in PBL before and after CyA therapy. The percent CD8+ cells in PBL are plotted individually or as the mean ± SD (O) before and after 12 weeks of CyA therapy. The normal value is 27.1 ± 7.5 based on analyses of 19 healthy individuals.

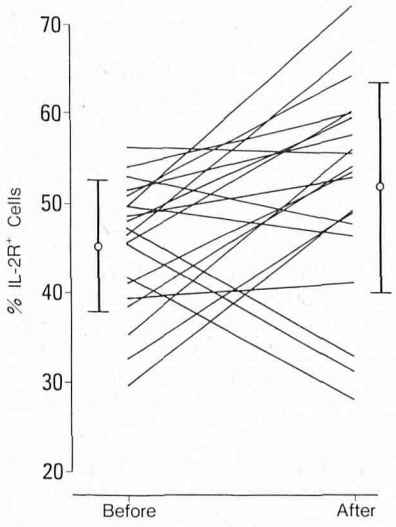

Figure 5. Capacities to express IL-2R of PBL in response to Con A stimulation before and after CyA. PBL were cultured for 48 h at 37°C in the presence of 10 μl/ml of Con A. The percent IL-2R+ cells in the culture are plotted individually or as the mean ± SD of 20 patients (O). The normal value is 44.5 ± 7.5 in 51 healthy individuals (28 males of 25 to 52 years old and 23 females of 24 to 46 years old).

patients with psoriasis or suppressed by CyA treatment, experiments were carried out in which a variety of T-cell functions were compared between patients and healthy volunteers both before and after therapy.

Because in vitro studies have revealed that the actions of CyA on T cells are mainly mediated by suppression of IL-2 production and IL-2 receptor (IL-2R) expression (reviewed in [31,32]), it was of particular interest to assess these steps of T-cell activation in our patients. A considerable variation was observed in the levels of IL-2 produced in response to Con A stimulation among the patients, but the mean IL-2 level in the 19 male patients (11.6 ± 5.8 U/ml) was not significantly different from that in 43 healthy male subjects (8.2 ± 5.0). Furthermore, the capacity to produce IL-2 was not suppressed after CyA treatment (11.0 ± 7.1) (Fig 4). Nor were any differences seen in IL-2R expression between patients with psoriasis (45.3 ± 7.3%) and 51 healthy individuals (44.5 ± 7.5) either before or after therapy (52.0 ± 11.6) (Fig 5). The proliferative response to PHA varied considerably among the 20 patients, but their average value of 43,000 ± 12,500 cpm was within the normal range of 50,100 ± 6,100 obtained with 200 healthy volunteers. Furthermore, this T-cell function remained unchanged after 12 weeks of CyA treatment (41,700 ± 9,400) (Fig 6). Nor was there any significant difference in spontaneous proliferation in the absence of exogenous stimulations, between the two groups either before or after therapy (data not shown).

Our patients were also studied for in vivo responsiveness to PPD, before and after therapy. Data in Fig 7 demonstrate that T-cell–mediated immunity was not changed by CyA treatment: 9.2 ±

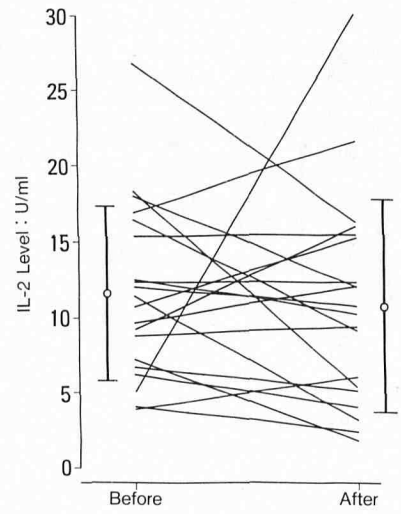

Figure 4. Capacities to produce IL-2 of PBL in response to Con A stimulation before and after CyA. PBL were cultured for 24 h at 37°C in the presence of 10 μg/ml of Con A. IL-2 levels (U/ml) in the culture supernatant are plotted individually or as the mean ± SD of 19 male patients (O). The normal value is 8.2 ± 5.0 in 43 healthy male individuals (20 to 61 years old).

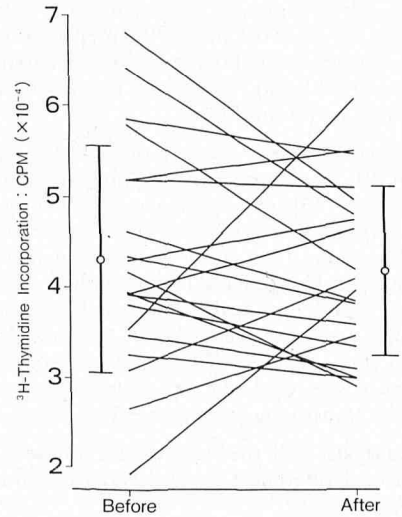

Figure 6. Proliferative responses of PBL to PHA stimulation before and after CyA therapy. PBL were cultured for 72 h at 37°C in the presence of 20 μg/ml of PHA. The values of ^{3}H-thymidine incorporation is plotted individually or as the mean ± SD (O). The normal value is 50,100 ± 6,100 in 200 healthy individuals (127 men of 20 to 68 years old and 73 women of 21 to 54 years old).

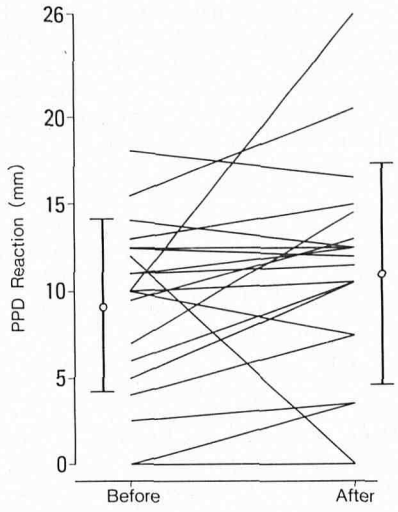

Figure 7. In vivo immune responsiveness to PPD before and after CyA therapy. The cutaneous inflammatory reaction was evaluated 48 h after intracutaneous injection of 0.1 ml of PPD solution. The mean diameters of erythematous areas with infiltration are plotted individually or as the mean ± SD (O). The values seen in the 20 patients are considered to be within the normal range for the general Japanese population.

5.0 mm before therapy and 11.0 ± 6.4 after therapy. These results suggest that patients with psoriasis have normal T-cell function and that the improvement in psoriasis observed with CyA is not merely due to a general or "systemic" suppression in T-cell functional activities.

Experiments were then undertaken to assess the possibility that patients with psoriasis have a genomic alteration(s) in T-cell–related genes and that this may contribute to their therapeutic response to CyA. Because the TcR plays a central role in antigen recognition and subsequent immune reactions (reviewed in [47]), our patients were examined for RFLP distribution of TcR β-chain genes. DNA samples from PBL of 10 healthy volunteers and the 20 patients with psoriasis were digested with Eco RI or Bam HI and analyzed by Southern blotting using cDNA of Cβ1. Eco RI digestion performed for each of the healthy individuals revealed two common restriction fragments, 12.0 and 4.2 kb (Fig 8), which is in agreement with previous studies [48,49] and corresponds to the germ-line configuration. We observed an additional fragment of $\simeq$9 kb in four of 10 healthy controls; this pattern of RFLP in the TcR β-chain has been previously reported [50]. This polymorphic fragment was also seen in only one (patient 1) of the 20 patients with psoriasis examined (Fig 8*A*,*B*). No other polymorphic fragment was observed in any of the healthy individuals or the patients. Bam HI digestion revealed one fragment of 24 kb in each of the healthy and psoriatic individuals (Fig 8*A*,*B*,*C*), which is also in agreement with previous studies [48,49]; this fragment is thought to be unrearranged TcR β-chain genes. No RFLP was observed in Bam HI fragments in any of the individuals tested.

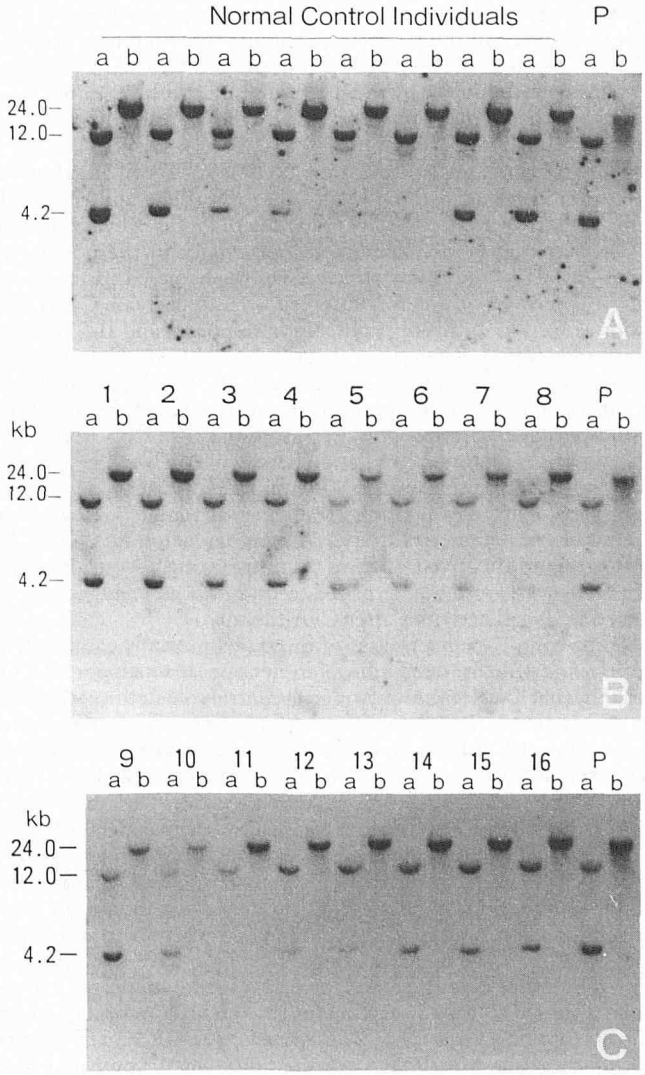

Figure 8. Southern hybridization analyses of the TcR β-chain gene in DNA from psoriasis patients and healthy individuals. DNA extracted from PBL of healthy individuals *(A)* or patients with psoriasis *(B* and *C)* were digested with Eco RI (a) or Bam HI (b) and examined by Southern gel analysis using Cβ1 as a probe. Human placenta DNA (Oncor) was electrophoresed as markers for germline bands (P). A polymorphic fragment of $\simeq$9 kb is noted in Eco RI digests in healthy individuals *(lanes 1, 5, 9,* and *11* in *A)* and in one patient (patient 1, *lane 1* in *B)*.

DISCUSSION

The present paper is the first to compare several phenotypic and functional activities of peripheral blood T cells in patients with psoriasis, before and after CyA therapy. Analyses included numbers and phenotypes of T cells, stepwise evaluations of T-cell activation by mitogen stimulation, and in vivo responsiveness to a foreign antigen. Despite the fact that the lesions of psoriasis improved significantly in response to CyA treatment in all our patients [44], no significant suppression was detected in any of the T-cell activities examined. Several investigators have hypothesized that CyA acts primarily on T cells in the treatment of psoriasis, based on their phenotypic analyses of lesional psoriatic skin [27,29,30]. Although there is some controversy over details, there seems to be a common understanding that both CD4+ and CD8+ cells are depleted from both lesional epidermis and dermis after several days to a few weeks of CyA administration [27,29,30,51,52]. We made the same observation as well in four of our patients by standard immunohistochemical staining (data not shown). These results, however, appear to be unsatisfactory for directly supporting their hypothesis, because CyA treatment also induces a rapid histologic improvement in epidermal hyperproliferation and other feature of psoriasis [24,30,44].

Sasaki et al have recently assessed the phenotypic alterations of peripheral blood T cells in patients with psoriasis undergoing CyA treatment [28]. These investigators failed to detect any significant changes in the CD4/CD8 ratio or in the percentages of T cells expressing various phenotypes, except for a slight decrease in helper inducer T cells by two-color analyses [28]. In contrast, Rubins and Merson reported a significant decrease in the CD4/CD8 ratio in psoriatic patients after treatment with the other immunomodulators, Thymalinum and Natrii nucleinas [53]. The conclusions drawn from our results and others is that a general or "systemic" suppression in T-cell activity may not necessarily accompany the

beneficial effect that is seen with CyA in psoriasis. On the other hand, we have not ruled out the possibility that CyA may influence "local" T-cell activities, within lesional psoriatic skin [54]. Our conclusion is supported by the recent finding that intralesional CyA has a beneficial effect on psoriasis, in the absence of significant systemic absorption [54,55].

The effect of CyA treatment on "systemic" T-cell activities has been monitored in patients with other disorders, and the results remain controversial, probably reflecting differences in background T-cell activities before therapy and difference in the dosage and duration of CyA administration. Assan et al studied patients with type 1 diabetes mellitus and observed CyA to decrease CD4/CD8 ratios as well as diminish proliferative responses and IL-2 production to PHA [56]. Khanuja et al studied renal transplant recipients following CyA therapy and found that CD4/CD8 ratios increased in patients with no rejection episodes, remained unchanged in those with reversible rejections, but decreased in those with irreversible rejections, suggesting a correlation between T-cell subset activities and clinical responsiveness [57]. In a more recent study on patients with type 1 diabetes mellitus, Müller et al found no changes in immunologic parameters after CyA therapy, including CD4/CD8 ratios and proliferative responses to mitogen and alloantigens [58]. Therefore, we were not surprised to find that no changes had occurred in T-cell activities after CyA therapy.

Comparing T-cell activities of untreated psoriasis patients with healthy individuals, our findings do not appear to support the hypothesis that T-cell immunity plays a central role in the pathogenesis of this disease. Based on differing findings from previous studies concerning the number and subsets of peripheral blood T cells in patients with psoriasis, considerable uncertainty remains. Several authors have demonstrated significant alterations of T-cell subsets [5,53], although others have not found any essential changes [2,59]. Concerning T-cell functional abnormalities, limited information is available; implicated are impaired suppressor T-cell [1] and helper T-cell [2] functions, a decrease in proliferative responses to mitogens [3], and an augumented proliferative responses to autologous epidermal cells [4]. These findings, however, have not been supported by other investigators. The present study indicates that peripheral blood T cells in patients with extensive psoriasis possess normal capacities to produce IL-2, to express IL-2R, and to proliferate in response to mitogen stimulation, when compared with healthy individuals. Furthermore, our results in genomic analyses failed to demonstrate any substantial changes in TcR β-chain genes in psoriatic individuals, compared with healthy individuals. Therefore, it cannot be concluded at present that there is a significant abnormality in "systemic" T-cell activities in patients with psoriasis. On the other hand, we have not ruled out the possibility that there exist an abnormality in "local" T cells within psoriatic tissue.

Recent advances in molecular biology have made it possible to study the mutations in T-cell–related functional genes, which may potentially contribute to the development of certain diseases [60]. RFLP distribution of TcR α- and β-chain genes has been correlated with susceptibility to several disorders, mainly autoimmune in nature, including type I diabetes mellitus [61], multiple sclerosis [62], myasthenia gravis [62], cystic fibrosis [63], rheumatoid arthritis [64], Hashimoto's thyroiditis [65], and Graves' disease [65]. However, lack of correlation has been reported with ulcerative colitis [66,67], Crohn's disease [66,67], and even with type I diabetes mellitus [68], multiple sclerosis [69], and myasthenia gravis [69]. With respect to psoriasis, no studies have reported the presence or the frequency of RFLP in TcR genes. In our studies, hybridization of Eco RI digests with Cβ1 cDNA revealed the presence of a polymorphic fragment of $\simeq$9 kb in one patient with psoriasis. Thus the frequency of this polymorphism was one in 20 of the psoriasis patients studied, and somehwat lower than that (four of 10) of the healthy individuals examined in this study. A similar pattern of polymorphism in Eco RI digests has been previously reported in two of 17 healthy individuals [50]. Thus, additional studies involving a larger number of patients and normal controls are clearly required. In our own laboratory, further studies are in progress examining genes of TcR α-, γ-, and δ-chains, IL-2 and IL-2R, which may reveal genomic abnormalities in T cells of individuals with this disease. Moreover, analyses of the genes encoding the functions of other cell types, impairment of which has contributed to psoriasis (as listed in the *Introduction*), should give further insights into the pathogenesis of this skin disorder.

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